Clontech® Laboratories, Inc.

SMARTer® Stranded RNA-Seq Kit User Manual

Cat. Nos. 634836, 634837, 634838, 634839
(080213)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

SMARTer cDNA Synthesis for the Illumina® Sequencing Platform

The **SMARTer Stranded RNA-Seq Kit** (Cat. Nos. 634836, 634837, 634838 & 634839) includes the components needed to generate indexed cDNA libraries suitable for next-generation sequencing (NGS) on any Illumina platform, starting from as little as 100 pg of polyA-purified or ribosomal RNA-depleted RNA. The kit consists of the SMARTer Stranded RNA-Seq Components, SeqAmp™ DNA Polymerase, and the llumina Indexing Primer Set (PCR primers for the amplification of indexed, paired-end Illumina-compatible sequencing libraries, which enable multiplexing of NGS library analysis).

The entire library construction protocol can be completed in less than 4 hr (Figure 1). The SMARTer Stranded RNA-Seq Kit utilizes our patented SMART™ (**S**witching **M**echanism **A**t 5' end of **R**NA **T**ranscript) technology, coupled with PCR amplification, to generate Illumina-compatible libraries without the need for enzymatic clean-up or adapter ligations. The directionality of the template-switching reaction preserves the strand orientation of the RNA, making it possible to obtain strand-specific sequencing data from the synthesized cDNA.

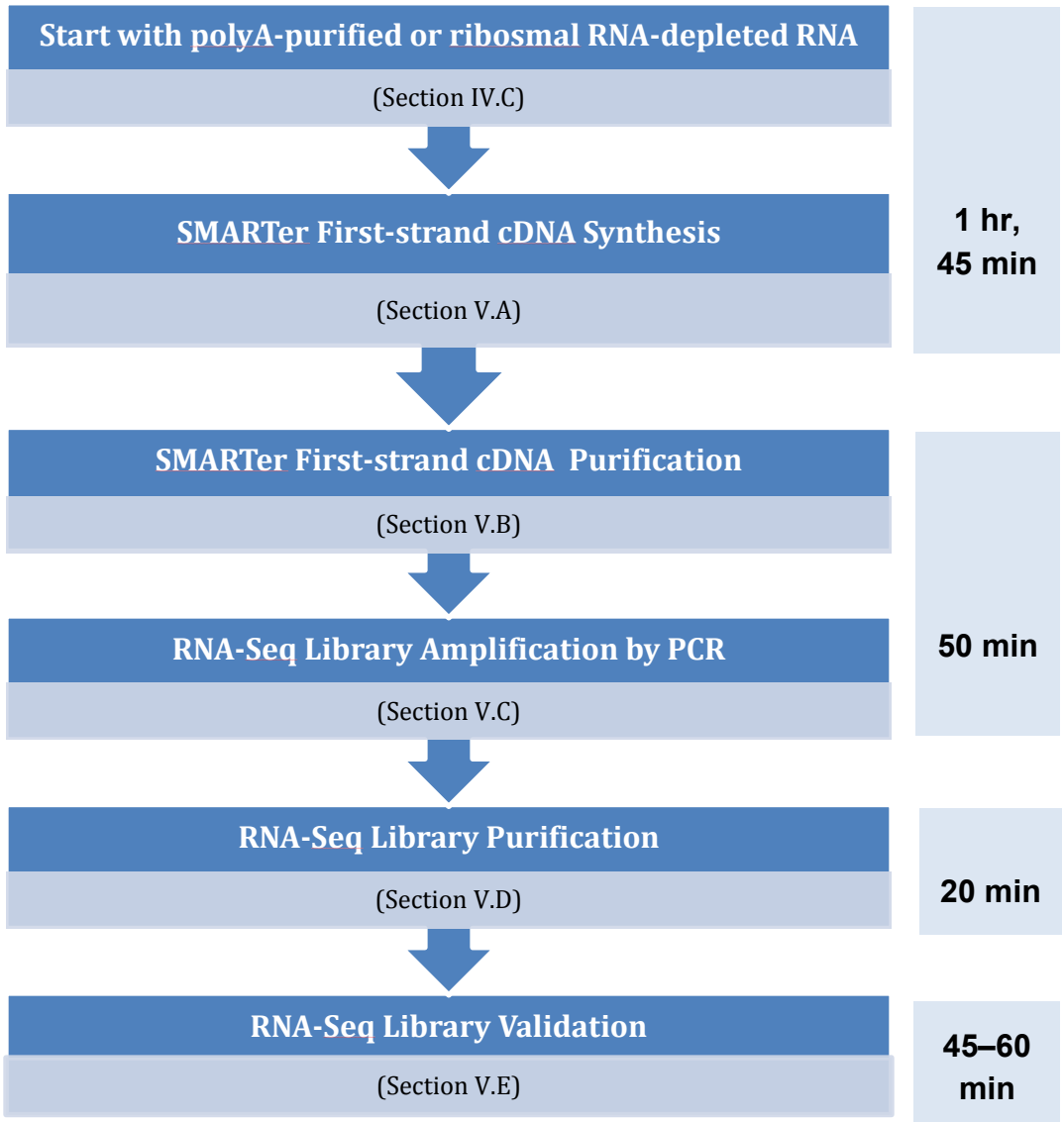

Figure 1. SMARTer Stranded RNA-Seq Kit protocol overview. You can complete this protocol in less than 4 hr.

The SMARTer Stranded RNA-Seq Kit starts with less than nanogram amounts of RNA. A modified N6 primer (the SMART Stranded N6 Primer) primes the first-strand synthesis reaction (Figure 2). For added simplicity, the RNA is chemically fragmented during denaturation.

NOTE: If your sample is degraded or of low quality, see Appendix A for a fragmentation-free protocol.

When SMARTScribe™ Reverse Transcriptase reaches the 5' end of the RNA fragment, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3' end of the cDNA. The carefully-designed SMARTer Stranded Oligo base-pairs with the non-template nucleotide stretch, creating an extended template to enable SMARTScribe RT continue replicating to the end of the oligonucleotide (Chenchik *et al.*, 1998). The resulting full-length, single-stranded (ss) cDNA contains the complete 5' end of the mRNA, as well as sequences that are complementary to the SMARTer Stranded Oligo.

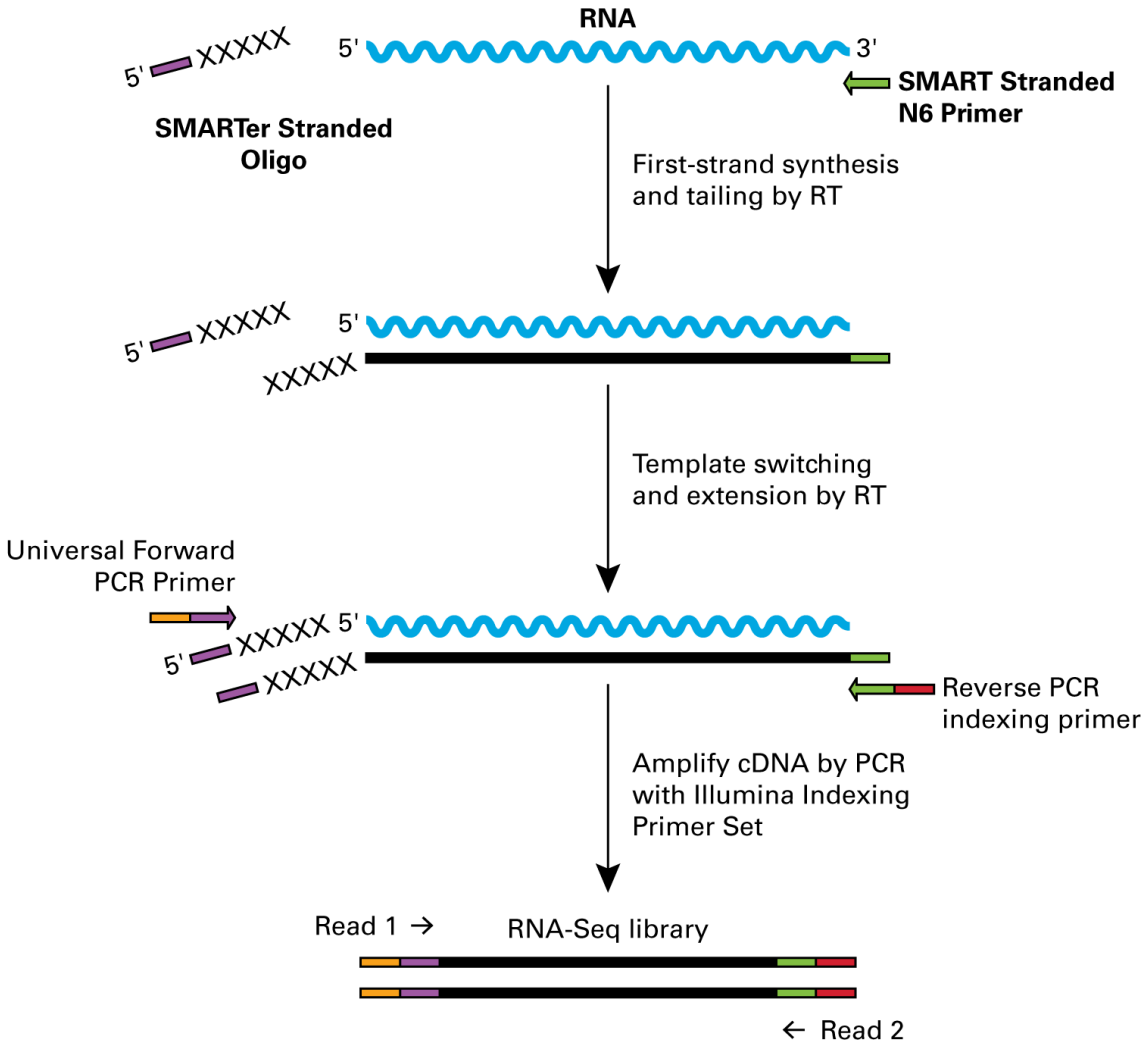

Figure 2. Flowchart of SMARTer Stranded RNA-Seq library generation.

II. List of Components

The following components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions. The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results.

The **SMARTer Stranded RNA-Seq Kits** (Cat. Nos. 634836, 634837, 634838 & 634839) consist of:

- **The SMARTer Stranded RNA-Seq Components** (Cat. No. 634840, 634841, 634842, or 634843)
- **The Illumina Indexing Primer Set** (Cat. No. 634844 or 634845), which contains PCR primers for the amplification of indexed, paired-end Illumina-compatible sequencing libraries.
- **SeqAmp DNA Polymerase** (Cat. No. 638504), a high fidelity, hot start PCR enzyme that is well-suited for use with the SMARTer Stranded RNA-Seq Kit for NGS. This optimized PCR enzyme has been shown to perform well even with challenging templates containing GC-rich and AT-rich regions.

The specific composition of each kit is as follows:

SMARTer Stranded RNA-Seq Kit (12 rxns, Cat. No. 634836)

- **12 rxns SMARTer Stranded RNA-Seq Kit**
  (Cat. No. 634840; Not sold separately)

  Box 1:

24 µl	SMARTer Stranded Oligo (12 µM)*
5 µl	Control Mouse Liver Total RNA (1 µg/µl)

  Box 2:

12 µl	SMART Stranded N6 Primer (12 µM)*
48 µl	5X First-Strand Buffer (RNase-Free)
24 µl	dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
12 µl	Dithiothreitol (DTT; 100 mM)
24 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
1 ml	Nuclease-Free Water
55 µl	RNase Inhibitor (40 U/µl)
240 µl	Stranded Elution Buffer

  * Clontech proprietary sequences.

- **12 rxns Illumina Indexing Primer Set** (Cat. No. 634844)

12 µl	Universal Forward PCR Primer (12.5 µM)
12 µl	Reverse PCR Primer Index 1 (12.5 µM)
12 µl	Reverse PCR Primer Index 2 (12.5 µM)
12 µl	Reverse PCR Primer Index 3 (12.5 µM)
12 µl	Reverse PCR Primer Index 4 (12.5 µM)
12 µl	Reverse PCR Primer Index 5 (12.5 µM)
12 µl	Reverse PCR Primer Index 6 (12.5 µM)
12 µl	Reverse PCR Primer Index 7 (12.5 µM)
12 µl	Reverse PCR Primer Index 8 (12.5 µM)
12 µl	Reverse PCR Primer Index 9 (12.5 µM)
12 µl	Reverse PCR Primer Index 10 (12.5 µM)
12 µl	Reverse PCR Primer Index 11 (12.5 µM)
12 µl	Reverse PCR Primer Index 12 (12.5 µM)

- **50 rxns SeqAmp DNA Polymerase** (Cat. No. 638504)

50 µl	SeqAmp DNA Polymerase
1.25 ml	SeqAmp PCR Buffer (2X)

SMARTer Stranded RNA-Seq Kit (24 rxns, Cat. No. 634837)

- **24 rxns SMARTer Stranded RNA-Seq Kit**
  (Cat. No. 634841; Not sold separately)

  Box 1:

48 µl	SMARTer Stranded Oligo (12 µM)*
5 µl	Control Mouse Liver Total RNA (1 µg/µl)

  Box 2:

24 µl	SMART Stranded N6 Primer (12 µM)*
96 µl	5X First-Strand Buffer (RNase-Free)
48 µl	dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
24 µl	Dithiothreitol (DTT; 100 mM)
48 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
1 ml	Nuclease-Free Water
55 µl	RNase Inhibitor (40 U/µl)
480 µl	Stranded Elution Buffer

  * Clontech proprietary sequences.

- **2 x 12 rxns Illumina Indexing Primer Set** (Cat. No. 634844)

12 µl	Universal Forward PCR Primer (12.5 µM)
12 µl	Reverse PCR Primer Index 1 (12.5 µM)
12 µl	Reverse PCR Primer Index 2 (12.5 µM)
12 µl	Reverse PCR Primer Index 3 (12.5 µM)
12 µl	Reverse PCR Primer Index 4 (12.5 µM)
12 µl	Reverse PCR Primer Index 5 (12.5 µM)
12 µl	Reverse PCR Primer Index 6 (12.5 µM)
12 µl	Reverse PCR Primer Index 7 (12.5 µM)
12 µl	Reverse PCR Primer Index 8 (12.5 µM)
12 µl	Reverse PCR Primer Index 9 (12.5 µM)
12 µl	Reverse PCR Primer Index 10 (12.5 µM)
12 µl	Reverse PCR Primer Index 11 (12.5 µM)
12 µl	Reverse PCR Primer Index 12 (12.5 µM)

- **50 rxns SeqAmp DNA Polymerase** (Cat. No. 638504)

50 µl	SeqAmp DNA Polymerase
1.25 ml	SeqAmp PCR Buffer (2X)

SMARTer Stranded RNA-Seq Kit (48 rxns, Cat. No. 634838)

- **48 rxns SMARTer Stranded RNA-Seq Kit**
  (Cat. No. 634842; Not sold separately)

  Box 1:

96 µl	SMARTer Stranded Oligo (12 µM)*
5 µl	Control Mouse Liver Total RNA (1 µg/µl)

  Box 2:

48 µl	SMART Stranded N6 Primer (12 µM)*
192 µl	5X First-Strand Buffer (RNase-Free)
96 µl	dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
48 µl	Dithiothreitol (DTT; 100 mM)
96 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
2 x 1 ml	Nuclease-Free Water
55 µl	RNase Inhibitor (40 U/µl)
960 µl	Stranded Elution Buffer

  * Clontech proprietary sequences.

- **48 rxns Illumina Indexing Primer Set** (Cat. No. 634845)

48 µl	Universal Forward PCR Primer (12.5 µM)
48 µl	Reverse PCR Primer Index 1 (12.5 µM)
48 µl	Reverse PCR Primer Index 2 (12.5 µM)
48 µl	Reverse PCR Primer Index 3 (12.5 µM)
48 µl	Reverse PCR Primer Index 4 (12.5 µM)
48 µl	Reverse PCR Primer Index 5 (12.5 µM)
48 µl	Reverse PCR Primer Index 6 (12.5 µM)
48 µl	Reverse PCR Primer Index 7 (12.5 µM)
48 µl	Reverse PCR Primer Index 8 (12.5 µM)
48 µl	Reverse PCR Primer Index 9 (12.5 µM)
48 µl	Reverse PCR Primer Index 10 (12.5 µM)
48 µl	Reverse PCR Primer Index 11 (12.5 µM)
48 µl	Reverse PCR Primer Index 12 (12.5 µM)

- **2 x 50 rxns SeqAmp DNA Polymerase** (Cat. No. 638504)

50 µl	SeqAmp DNA Polymerase
1.25 ml	SeqAmp PCR Buffer (2X)

SMARTer Stranded RNA-Seq Kit (96 rxns, Cat. No. 634839)

- **96 rxns SMARTer Stranded RNA-Seq Kit**
  (Cat. No. 634843; Not sold separately)

  Box 1:

192 µl	SMARTer Stranded Oligo (12 µM)*
5 µl	Control Mouse Liver Total RNA (1 µg/µl)

  Box 2:

96 µl	SMART Stranded N6 Primer (12 µM)*
384 µl	5X First-Strand Buffer (RNase-Free)
192 µl	dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
96 µl	Dithiothreitol (DTT; 100 mM)
192 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
3 x 1 ml	Nuclease-Free Water
55 µl	RNase Inhibitor (40 U/µl)
2 ml	Stranded Elution Buffer

  * Clontech proprietary sequences.

- **2 x 48 rxns Illumina Indexing Primer Set** (Cat. No. 634845)

48 µl	Universal Forward PCR Primer (12.5 µM)
48 µl	Reverse PCR Primer Index 1 (12.5 µM)
48 µl	Reverse PCR Primer Index 2 (12.5 µM)
48 µl	Reverse PCR Primer Index 3 (12.5 µM)
48 µl	Reverse PCR Primer Index 4 (12.5 µM)
48 µl	Reverse PCR Primer Index 5 (12.5 µM)
48 µl	Reverse PCR Primer Index 6 (12.5 µM)
48 µl	Reverse PCR Primer Index 7 (12.5 µM)
48 µl	Reverse PCR Primer Index 8 (12.5 µM)
48 µl	Reverse PCR Primer Index 9 (12.5 µM)
48 µl	Reverse PCR Primer Index 10 (12.5 µM)
48 µl	Reverse PCR Primer Index 11 (12.5 µM)
48 µl	Reverse PCR Primer Index 12 (12.5 µM)

- **2 x 50 rxns SeqAmp DNA Polymerase** (Cat. No. 638504)

50 µl	SeqAmp DNA Polymerase
1.25 ml	SeqAmp PCR Buffer (2X)

Storage Conditions:

- Store Control Mouse Liver Total RNA and SMARTer Stranded Oligo at –70°C.
- Store Stranded Elution Buffer at –20°C. Once thawed, the buffer can be stored at Room Temperature.
- Store all other reagents at –20°C.

III. Additional Materials Required

The following reagents are required but not supplied. These materials have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results:

- Single channel pipette: 10 µl, 20 µl and 200 µl, one each
- Filter pipette tips: 10 µl, 20 µl and 200 µl, one box each
- One QuickSpin minicentrifuge for 0.2 ml tubes

For PCR Amplification & Validation:

- One dedicated PCR thermal cycler used only for first-strand synthesis
- High Sensitivity DNA Kit (Agilent, Cat No. 5067-4626)
- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Cat. No.1402-4700)
- Nuclease-free nonsticky 1.5 ml tubes (USA Scientific, Cat. No. 1415-2600)

For SPRI Bead Purification:

- Agencourt AMPure PCR Purification Kit (5 ml Beckman Coulter, Part No. A63880; 60 ml Beckman Coulter, Part No. A63881).
- Magnetic separation device for 0.2 ml tubes (see Appendix B)

  NOTE: We strongly recommend using separate magnets for purification of first-strand cDNA (Section V.B) and purification of the RNA-Seq library (Section V.D) to prevent cross contamination.

- 80% ethanol

IV. General Considerations

A. Recommendations for Preventing Contamination

1. **Before you set up the experiment, it is advisable to have two physically separated work stations:**

    - **A PCR Clean Work Station** for all pre-PCR experiments that require clean room conditions such as first-strand cDNA synthesis (Section V.A) and purification of first-strand cDNA (Section V.B).
    - **A second work station located in the general laboratory** where you will perform PCR to amplify the RNA-Seq library (Section V.C), purify the RNA-Seq library (Section V.D), and measure its concentration (Section V.E).

    IMPORTANT: The PCR work station must be located in a clean room with positive air flow, as contamination may occur very easily. Once contamination occurs it can be difficult to remove.

2. **Guidelines for clean room operation:**

    - Only move materials/supplies from the clean room to the general lab, NOT the other way around. Don't share any equipment/reagents between the clean room and the general lab.
    - Use a separate PCR machine inside the PCR workstation for cDNA synthesis.
    - Wear gloves and sleeve covers throughout the procedure to protect your RNA samples from degradation by contaminants and nucleases. Be sure to change gloves and sleeve covers between each section of the protocol.

B. General Requirements

- **The success of your experiment depends on the quality of your starting RNA sample. Prior to cDNA synthesis, please make sure that your RNA is free of contaminants.**
- The assay is very sensitive to variations in pipette volume, etc. Please make sure that all your pipettes are calibrated for reliable delivery, and that nothing is attached to the outside of the tips.
- All lab supplies related to SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMARTer cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount of enzyme added or the concentration of DNA in the reactions. The amounts and concentrations have been carefully optimized for the SMARTer amplification reagents and protocol.
- If you are using this protocol for the first time we strongly recommend that you perform negative and positive control reactions to verify that kit components are working properly.

C. Sample Preparation

The sequence complexity and the average length of SMARTer cDNA is noticeably dependent on the quality of starting RNA material.

- There are several commercially available products that enable purification of total RNA preparations from extremely small samples [e.g. Clontech offers the **NucleoSpin RNA XS Kit** (Cat. No. 740902.10) for purification of RNA from 10^2 cells].
- When choosing a purification method (kit), ensure that it is appropriate for your sample amount.

D. Sample Requirements

Ribosomal RNA (rRNA) depletion

We strongly recommend removing rRNA prior to cDNA synthesis with the SMARTer Stranded RNA-Seq Kit. If your sample has not been depleted of rRNA, you may not obtain sufficient reads for analysis, and any results you do obtain may be compromised.

Input RNA length

- The SMARTer Stranded RNA-Seq Kit was developed for full-length, intact RNA. An alternative protocol for degraded samples is available in Appendix A. You may need to adjust the fragmentation times for RNA of intermediate lengths.
- After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA quality using the **Agilent RNA 6000 Pico Kit** (Cat. No. 5067-1513).

Input RNA purity and quantity

The input amounts indicated in this kit are for poly(A)-purified, rRNA-depleted, or otherwise purified RNA samples.

- **Purity of input RNA:** Input RNA should be free from genomic or carrier DNA, and contaminants that would interfere with oligo annealing or reverse transcriptase reactions.

  IMPORTANT: Purified total RNA should be resuspended in nuclease-free water, **not in TE or other buffers containing EDTA.** Chelation of divalent cations by EDTA will interfere with RNA fragmentation.

- **Volume and amount of input RNA:** This kit accommodates up to 8 µl of input RNA. This protocol has been optimized for cDNA synthesis starting from 1 ng of RNA. However, if your RNA sample is not limiting, we recommend that you start with more than 1 ng of RNA.

E. Sequencing Analysis Guidelines

- The first three bases of the first sequencing read (Read 1) are derived from the SMARTer Stranded Oligo. We recommend trimming these bases prior to mapping.
- Read 1 is derived from the sense strand of the input RNA. If you are performing paired-end sequencing, Read 2 corresponds to the antisense strand.

V. Protocols

A. PROTOCOL: First-Strand cDNA Synthesis

During this step, RNA is fragmented and converted to single-stranded (ss) cDNA that contains sequences complementary to the SMARTer Stranded Oligo.

IMPORTANT:

- **The following protocol is designed for full-length, undegraded RNA.** The first two steps will simultaneously fragment and prime the RNA for cDNA synthesis.

  NOTE: This protocol was designed to fragment full-length polyadenylated RNA for a final mean library insert size of ~180 bp. For some RNA samples or sequencing applications, it may be appropriate to titrate the fragmentation time to achieve optimal yield and library size.

- **When working with degraded RNA samples, such as FFPE RNA,** use the First-Strand cDNA Synthesis Protocol for Degraded Samples in Appendix A instead, because additional fragmentation is unnecessary, and will result in lower library yields.

1. Mix the following components on ice:

1–8 µl	RNA (1–100 ng)
1 µl	SMART Stranded N6 Primer (12 µM)
4 µl	5X First-Strand Buffer (RNase-Free)
0–7 µl	Nuclease-Free Water
13 µl	Total volume

2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler for 5 min, then place the samples on ice for 2 min.

   NOTE: Steps 4–6 should not be delayed after completing Step 2, since they are critical for first-strand cDNA synthesis. You can prepare your master mix, except for SMARTScribe Reverse Transcriptase, (for Step 3), while your tubes are incubating (Step 2) in order to jump start the cDNA synthesis.

3. Prepare enough Master Mix for all reactions, plus 10%, by combining the following reagents in the order shown on ice.

0.5 µl	DTT (100 mM)
0.5 µl	RNase Inhibitor
2 µl	dNTP Mix (10 mM)
2 µl	SMARTer Stranded Oligo (12 µM)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
7 µl	Total volume per reaction

   * Add the reverse transcriptase to the master mix immediately prior to use. Mix well by gently vortexing and spin the tubes briefly in a microcentrifuge.

4. Add 7 µl of the Master Mix to each reaction tube from Step 2. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes in a preheated thermal cycler at 42°C for 90 min.
6. Terminate the reaction by heating the tubes at 70°C for 10 min, then leave them in the thermal cycler at 4°C until the next step (Section V.B.1).

   NOTE: If desired, you may stop here and store the reaction tubes at 4°C overnight before proceeding to Section V.B.

B. PROTOCOL: Purification of First-Strand cDNA using SPRI AMPure Beads

The first-strand cDNA selectively binds to SPRI beads leaving contaminants in solution which is removed by a magnetic separation. The beads are then directly used for RNA-Seq library amplification.

NOTES:

- Aliquot SPRI beads and allow them to come to room temperature for 30 min prior to use.
- Before use, beads should be brought to room temperature and mixed well to disperse.
- You will need a Magnetic Separation Device for 0.2 ml tubes. If you do not have such a device, we recommend constructing one using the instructions in Appendix B.
- Clean-up of SMARTer reactions must be performed using Ampure XP beads. **Spin columns do not adequately remove adapter-dimers from the reactions and will result in experimental failure!**

To purify the SMART cDNA from unincorporated nucleotides and small (< 100 bp) cDNA fragments, follow this procedure for each reaction tube:

1. Add 20 µl of SPRI AMPure beads to each sample using a 20 µl pipetter.
    - Mix by vortexing for 5 sec or by pipetting the entire volume up and down at least 10 times.
    - The beads are viscous; suck the entire volume up, and push it out slowly.
2. Incubate at room temperature for 8 min to let DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the walls of the tube. Place the sample tubes on the Magnetic Separation Device for 5 min or longer, until the solution is completely clear.
4. While the tubes are sitting on the magnetic stand, pipette out the supernatant.
5. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads, in order to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant.
6. Repeat Step 5.
7. Perform a brief spin of the tubes (~2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for ~3–5 min until the pellet appears dry. You may see a tiny crack in the pellet.

    NOTE: Under- or over-drying the beads will reduce PCR efficiency, resulting in lower yields.
9. If using more than 12 PCR cycles (<10 ng input RNA), elute the cDNA in 20 µl of Nuclease-Free Water, transfer to a fresh 0.2 ml tube, and repeat Steps 1–8 to ensure complete removal of adapter dimers. Otherwise proceed immediately to Section V.C.
    - Add 20 µl Nuclease-Free Water to the pellet.
    - Thoroughly resuspend the beads and allow to rehydrate for 2 min.
    - Briefly spin the sample tubes to collect the liquid from the walls of the tube. Place the sample tubes on the Magnetic Separation Device for 1 min or longer, until the solution is completely clear.
    - Transfer the supernatant to a fresh 0.2 ml tube

C. PROTOCOL: RNA-Seq Library Amplification by PCR

The purified first-strand cDNA is amplified into RNA-Seq Libraries using SeqAmp DNA Polymerase, the Universal Forward PCR Primer, and the Reverse PCR Primers from the Illumina Indexing Primer Set.

IMPORTANT: Optimal parameters may vary with different templates and thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles.

Table 1. Cycling Guidelines Based on Amount of Starting Material

Amount of Input RNA (ng)	Typical Number of PCR Cycles
1	16
10	12
100	9

1. Prepare a PCR Master Mix for all reactions. Separate master mixes should be prepared for different library indexes. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a microcentrifuge:

25 µl	2X SeqAmp PCR Buffer
1 µl	Universal Forward PCR Primer (12.5 µM)
1 ul	Reverse PCR Primer* (12.5 µM)
1 µl	SeqAmp DNA Polymerase
22 µl	Nuclease-Free Water
50 µl	Total volume per reaction

*Your selection of the Reverse PCR Primer will determine which of the 12 indexing sequences in the Illumina Indexing Primer Set will be associated with your library.

2. Add 50 µl of PCR Master Mix to each tube containing DNA bound to the beads from Section V.B., Step 8. Mix well, making sure that the beads are uniformly resuspended.

3. Place the tube in a preheated thermal cycler with a heated lid. Start thermal cycling using the following program:

94°C		1 min
X[a] cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C		forever

[a] The number of cycles depends on the amount of input RNA. See Table 1 (above) for guidelines.

D. PROTOCOL: Purification of the RNA-Seq Library using SPRI AMPure Beads

The amplified RNA-Seq library is purified by immobilizing it onto SPRI beads. The beads are then washed with 80% ethanol and eluted in Stranded Elution Buffer.

1. Add 50 µl of SPRI AMPure beads to each sample.
    - Mix by vortexing 5 sec or by pipetting the entire volume up and down at least 10 times.
    - The beads are viscous; suck the entire volume up, and push it out slowly.
2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the Magnetic Separation Device for 5 min or longer, until the solution is completely clear.
4. While the tubes are sitting on the magnetic stand, pipette out the supernatant.
5. Keep the tubes on the magnetic stand. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 one more time.
7. Perform a brief spin of the tubes (~2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for ~3–5 min until the pellet appears dry. You may see a tiny crack in the pellet.

    NOTE: Be sure to dry the pellet enough.
    - If you under-dry the pellet, ethanol will remain in the sample tubes. The ethanol will reduce your RNA-Seq library recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet, it will take longer than 2 min to rehydrate (Step V.D.9).
9. Once the beads are dried, add 20 µl of Stranded Elution Buffer to cover the beads. Remove the tubes from the magnetic stand and mix thoroughly to resuspend the beads.

    NOTE: Be sure the beads are completely resuspended. The beads can sometimes stick to the sides of the tube. We recommend vortexing or directly pipetting the beads up and down to ensure complete dispersion.
10. Incubate at room temperature for 2 min to rehydrate.
11. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the Magnetic Separation Device for 1 min or longer, until the solution is completely clear.
12. Transfer the clear supernatant containing the purified RNA-Seq library from each tube to a nuclease-free nonsticky tube.

E. PROTOCOL: Validation Using the Agilent 2100 BioAnalyzer

1. Dilute 1 µl of the amplified RNA-Seq library with 3 µl Stranded Elution Buffer.
2. Use 1 µl of the diluted sample for validation using the Agilent 2100 BioAnalyzer and the High Sensitivity DNA Chip from Agilent's **High Sensitivity DNA Kit** (Cat. No. 5067-4626). See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
3. Compare the results for your samples and (and controls, if performed) to determine whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative control (Figure 3, Panel B), and a distinct peak spanning 150–1,000 bp, peaked at ~300 bp for the positive control RNA sample (Figure 3, Panel A), yielding > 7.5 nM RNA-Seq Library (depending on the input and number of cycles).

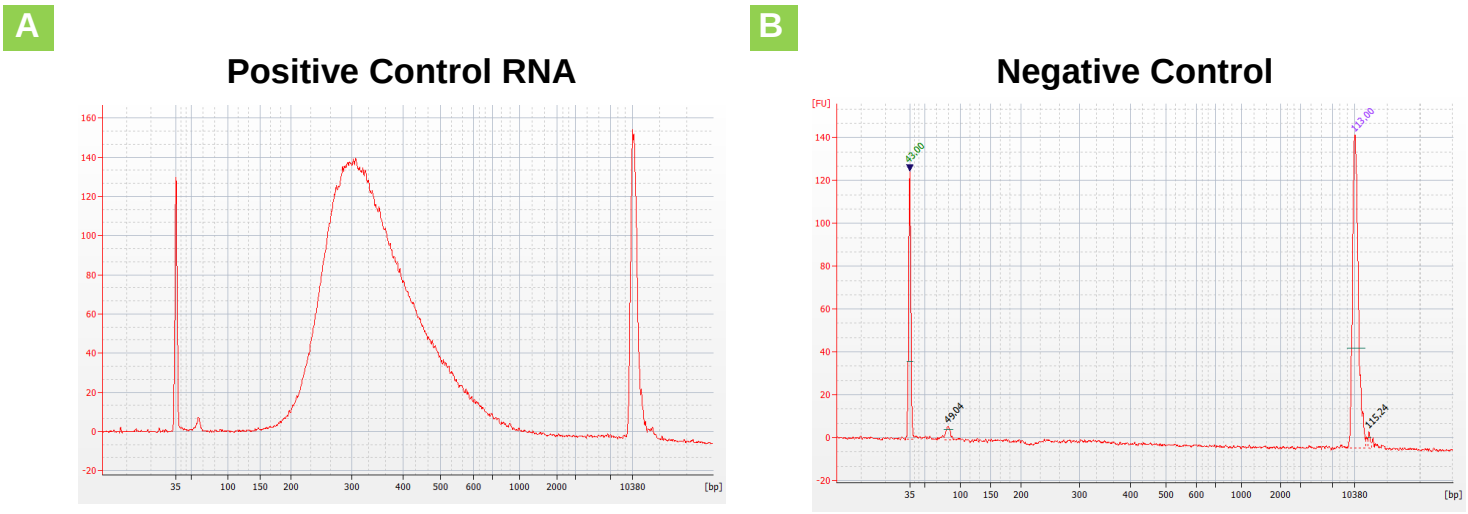

Figure 3. Electropherogram example results from Agilent 2100 bioanalyzer.

VI. References

Chenchik, A. et al. (1998). In *RT-PCR Methods for Gene Cloning and Analysis*. (BioTechniques Books, MA), pp. 305-319.

Appendix A: First-Strand cDNA Synthesis Protocol for Degraded Samples

Our typical protocol for first-strand cDNA synthesis (Section V.A) includes simultaneous RNA fragmentation. If your RNA is already fragmented, use this alternative protocol for first-strand cDNA synthesis.

1. Mix the following components on ice:

1–8 µl	RNA (1–100 ng)
1 µl	SMART Stranded N6 Primer (12 µM)
0–7 µl	Nuclease-Free Water
9 µl	Total volume

2. Incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min, then put the samples on ice for 2 min.

NOTE: Steps 4–6 should not be delayed after completing Step 2, since they are critical for first-strand cDNA synthesis. You can prepare your master mix, except for SMARTScribe Reverse Transcriptase, (for Step 3), while your tubes are incubating (Step 2) in order to jump start the cDNA synthesis.

3. Prepare enough Master Mix for all reactions, **plus 10%, by combining the following reagents in the order shown on ice.**

4 µl	5X First Strand Buffer
0.5 µl	DTT (100 mM)
0.5 µl	RNase Inhibitor
2 µl	dNTP Mix (10 mM)
2 µl	SMARTer Stranded Oligo (12 µM)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
11 µl	Total volume per reaction

* Add the reverse transcriptase to the master mix immediately prior to use. Mix well by gently vortexing and spin the tubes briefly in a microcentrifuge.

4. Add 11 µl of the Master Mix to each reaction tube from Step 2. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes in a preheated thermal cycler at 42°C for 90 minutes.
6. Terminate the reaction by heating the tubes at 70°C for 10 min, then leave them in the thermal cycler at 4°C until the next step (Section V.B.1).

NOTE: If desired, you may stop here and store the reaction tubes at 4°C overnight before proceeding to Section V.B.

Appendix B: Constructing a Magnetic Separation Device for 0.2 ml PCR Tubes

It can be difficult to find magnetic separation devices designed specifically to handle 0.2 ml PCR strip tubes. Often, one can place strip tubes in a column/row of a magnetic separation device designed for use with 96-well plates. Alternatively, one can construct a suitable low-cost separation device from common laboratory materials.

Example 1: Using a 96-well separation device with strip tubes

As seen in Figure 4, you may place the tubes in the top part of an inverted P20 or P200 Rainin Tip Holder which is taped to a MagnaBlot II Magnetic Separator (Promega Part No. V8351)

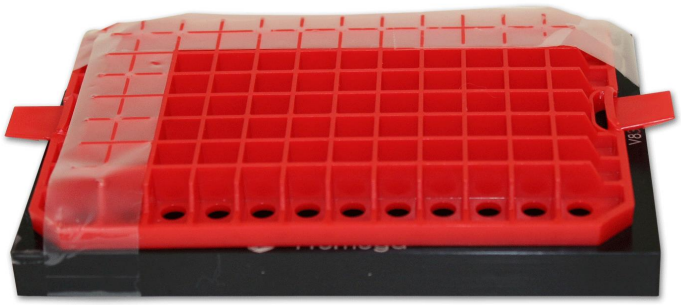

Figure 4. Setup for positioning 0.2 ml tubes containing first-strand cDNA on a MagnaBlot II Magnetic Separator.

Example 2: Building a 0.2ml tube magnetic separation device from rare earth bar magnets and a tip rack

As seen in Figure 5, neodymium bar magnets are taped together on the underside of the top section of a 20 µl tip rack (Panel A), and the rack is inverted so the tubes can be inserted (Panel B).

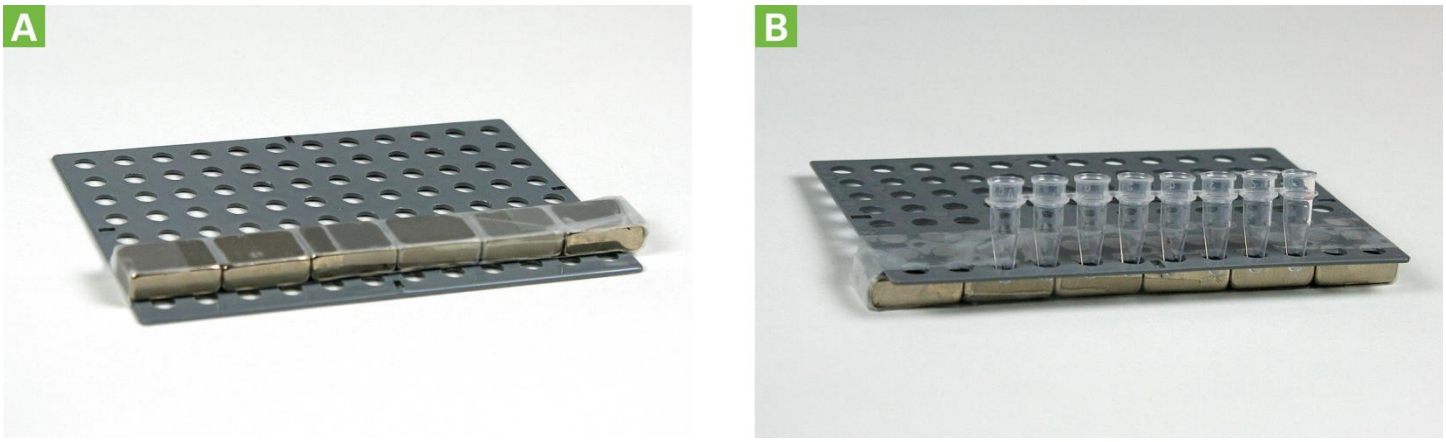

Figure 5. Constructing a magnetic separation device for 0.2 ml tubes from rare earth magnets. **Panel A** shows six 0.75" x 0.25" x 0.5" neodymium bar magnets (Applied Magnets Model # NB026) taped together on the underside of the top section of a 20 µl tip rack. **Panel B** shows the upright rack, into which an 8-tube strip of 0.2 ml tubes has been inserted.

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